Characterization of biofilm forming acidotolerant *Lysinibacillus* sp. RTA 01 and its utilization in biosynthesis of chromium oxide nanoparticles

Thesis submitted to

National Institute of Technology, Rourkela

In partial fulfilment for the degree of Master of Science in Life Science

Submitted By

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2015

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RIGHT TO INFORMATION

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Assistant Professor

May 12, 2015

CERTIFICATE

This is to certify that the project report entitled **"Characterization of biofilm forming acidotolerant *Lysinibacillus* sp. RTA 01 and its utilization in biosynthesis of chromium oxide nanoparticles"** submitted by **Ms. Laxmi Priya Mishra** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in **LIFE SCIENCE** is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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ACKNOWLEDGEMENT

I express my deepest gratitude and reverence to my advisor Dr. Surajit Das, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela for his keen interest, inspiring guidance, strict supervision and constant support throughout the entire project work which made this thesis reality.

I would like to thanks to Dr. Sujit Kumar Bhutia, HOD, Department of Life Science, National Institute of Technology, Rourkela for his constant support.

I am thankful to all the faculty members of the Department Of Life Science, National Institute of Technology, Rourkela for constant guidance and extending all the facilities for successfully completing the same.

It gives immense pleasure to express my heartfelt thanks to Mr. Rituraj and Mr. Himansu Kumar PhD scholars, Laboratory of Environmental and Microbial Ecology, (LEnME) Department of Life Science, National Institute of Technology, Rourkela for their constant support and moral advice. I am thankful to all lab members of LEnME for valuable and kind support during my project work.

I express my unfathomable love and affection and blissful blessings to all my loving friends for their contribution and indebtedness to my beloved parents for their love and immense support , I pray that their blessing will always be with me as my weapon and company in every walk of my life.

Lastly , with all my devotion I pray to almighty that has always been so kind to me and has always blessed me and helped me throughout.

Laxmi Priya Mishra

413LS2038

DECLARATION

I, Laxmi Priya Mishra hereby declare that, this thesis entitled "Characterization of biofilm forming acidotolerant *Lysinibacillus* sp. RTA 01 and its utilization in biosynthesis of chromium oxide nanoparticles" has been written by me, and submitted to National Institute of Technology , Rourkela for the partial fulfillment of the requirement for the degree of Master of Science (Life Science) is an authentic record of the work carried out by me, under the guidance of Dr. Surajit Das, Department of Life Science, National Institute of Technology, Rourkela and that any part of the result has not been presented for the award of any degree, diploma or any equivalent qualification.

Laxmi Priya Mishra
Roll no. 413LS2038

Date: 12.05.2015
Place: Rourkela

List of Abbreviation and Symbol

NPs	Nanoparticles
Cr_2O_3	Chromium Oxide
$K_2Cr_2O_7$	Potassium dichromate
Cd $(NO_3)_2$	Cadmium Nitrate
$ZnSO_4$	Zinc Sulphate
Pb $(NO_3)_2$	Lead Nitrate
EPS	Extracellular Polymeric Substance
MIC	Minimum Inhibitory Concentration
rpm	Revolution per Minute
FESEM	Field Emission Scanning Electron Microscope
XRD	X-Ray Diffraction
FTIR	Fourier Transformed Infra-red Spectroscopy
ATR	Attenuated Total Reflectance
BCP NP	Bacterial Cell pellet Nanoparticle
BCS NP	Bacterial Cell Supernatant Nanoparticle
BS NP	Bacterial Supernatant Nanoparticle
EPS NP	Extracellular polymeric Substance Nanoparticle
LB	Luria Bertani Broth
NB	Nutrient Broth
BHB	Bushnell Haas Broth
M	Molar
H	Hour
θ	Theta
µl	Microliter

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Abstract

In this rapidly developing world with the increase in development there is increase in the toxic waste also. The problem has become so alarming that the toxic waste management requires an urgent attention. Although many conventional techniques has been utilized for waste remediation but recently developed nanotechnology based remediation process has strengthen the remediation process. Here we report the ability of *Lysinibacillus* sp. RTA 01 isolated from contaminated sites for the biological synthesis of chromium oxide (Cr_2O_3) nanoparticles (NPs) which provide us for the development of clean, non-toxic and environment friendly procedure for NPs synthesis. The synthesized NPs from different parts of isolate like cell pellet, supernatant and EPS were characterized by UV Visible spectrophotometer, XRD (X-Ray Diffraction), FTIR (Fourier Transform Infra-red Spectroscopy), FESEM (Field Emission Scanning Electron Microscope), for confirming the formation and size of NPs. Zeta analysis was also done for determining the stability of the synthesized NPs. The bacterial isolate showed more than 50% reduction of hexavalent chromium (Cr^{6+}) to trivalent chromium (Cr^{3+}) when incubated with media supplemented with $K_2Cr_2O_7$ solution. The reduction of toxic Cr^{6+} into stabilized Cr^{3+} NPs by acidotolerant *Lysinibacillus* sp. RTA 01 showed the large scale industrial application in successful detoxification of contaminated sites in an environmentally sustainable way.

Key Words: acidotolerant, *Lysinibacillus*, biofilm-EPS, chromium, bio-reduction, Cr_2O_3 nanoparticle

Introduction

Industrialization, urbanization and development are two sides of a coin. In the developed country like India, with increase in industrialization and urbanization there is an increase in the production of toxic waste which has become a major problem now days. Environmental pollution is an increasing hazard to human health and it is more severe in the industrial intense cities. Human beings manipulate the environment or even the entire ecosphere by changing the global cycles of elements or by releasing chemicals, industrial effluents and pesticides in the environment; such adopted ecosphere possess threat to man's survival on the earth. With the increase in the use of metals in our day to day life, the exploitation of nature has also increased with the increase in concentration of heavy metals in the ecosystem which adds toxicity to the environment.

Chromium (Cr) is the most abundant of the Group VIA family of metallic elements present at a concentration of nearly 400 parts per million in the earth's crust as various minerals (Table 1). It is the 13th most common element of earth. It is one of the world's most critical and highly soluble metal pollutants having wide range of uses in the metals and chemical industries (Kotas et al., 2000). Cr exists in the environment in several diverse forms, among which hexavalent chromium Cr^{6+} is a carcinogenic, a potential soil, surface water and ground water contaminant. A slight elevated level of Cr^{6+} causes environmental and health problems because of its high toxicity, mutagenicity and carcinogenicity. However, its reduced trivalent form, (Cr^{3+}) is less toxic, insoluble and a vital nutrient for humans in small quantity (Cheung et al., 2007).

Table 1. Comprehensive data on Chromium

(EPA, United states Environment protection agency, 2013**).**

Name, Symbol, Number	Chromium, Cr, 24
Group, Period, Block	6,4 ,d
Element Category	Transition metal
Atomic Weight	$51.9961(6)gmol^{-1}$
Electronic Configuration	[Ar] $3d^5 4s^1$
Oxidation state	6, 5, 4, 3, 2, 1 ,-1 and -2
Atomic radius	128 pm

There has been an increasing demand for chromate ores in recent years due to its use in stainless steel and other alloy. In the open cast mining processes the chromite ore as well as waste rock material are dumped in the open ground without consolidating the environmental aspects, which causes damage to the topography of that area and leaching of Cr^{6+} and other impurities to the ground water as well as surface water bodies (Tiwari et al., 2005). Not only ore mining but also effluents from textile, leather, tannery, electroplating, galvanizing dyes and pigments, metallurgical and paint industry and other metal processing and refining operations at small and large scale sector contain considerable amount of Cr causing problem for both aquatic and human population (Baysal et al., 2013). The problem has become so alarming calling for an urgent need for remediation of the Cr^{6+} contaminated sites for safety of lives of people living nearby. There are several chemical methods utilized for detoxification of Cr^{6+} such as chemical precipitation, oxidation, reduction, filtration, ion exchange, electrochemical treatment, membrane technology, reverse osmosis and evaporation recovery but these techniques have not been utilized in a beneficial way because they are expensive and sometimes adds other impurities creating additional problem for existing environment (Kieu et al., 2011). However, biological treatment using microorganisms, which provides reliable, nontoxic method, is an inexpensive technology available for heavy metal contaminated sites. Microorganisms have developed survival strategies in heavy metal polluted habitats. They have different detoxifying mechanism such as bioaccumulation, biosorption, biotransformation and bio mineralization; these could be used to design economical bioremediation process for remediation of heavy metal toxicity (Morcillo et al., 2014). In terms of biosorption polysaccharides, proteins and lipids on bacterial cell wall offer many functional groups such as carboxylate, hydroxyl, phosphate, amino and sulfate groups which can bind metal ions (Wang et al., 2009). Taking benefit of this natural affinity of compounds for heavy metals one could successfully utilize the microbes for purification of metal contaminated sites. In addition to this, microbes utilizing the toxic metals were able to produce nanoparticles (NPs) increasing the capacity of adsorption.

Nanoremediation is a recent and emerging technology for remediation of toxic metals. NPs have unique property of high surface area to volume ratio makes it highly reactive. As it is having more number of reactive sites this increases contact with contaminants leading to rapid reduction of contaminates resulting in better, cheaper and faster site cleanup (Nirmala et al., 2013). The NPs can be used profitably for in situ applications. Due to its minute size they can

easily enter small spaces in the subsurface allowing particles to travel farther distance than the larger macro particles and remain suspended in the ground water (Tratnyek et al., 2006). A variety of chemical and physical and hybrid methods have been used for synthesis of NPs. Although the physical and chemical methods are widely used now days, but these are associated with problems of toxicity, cost, high energy consumption and production of hazardous waste (Li et al., 2011). As an alternative, utilizing biological method of NPs synthesis which could be ecofriendly, low cost and energy efficient compared to physical and chemical methods. Nanotechnological products called often as a technology of the future used in various fields such as electronics, computer technology, information technology, cosmetics industry, agriculture, textile, defense, pharmaceuticals and environment due to its unique properties compared to its bulk material (Bualzea et al., 2007). These practical applications explain why nanomaterials are arousing great interest in the scientific and economic sphere. Wide application of NPs in biological field has been represented in Fig 1.

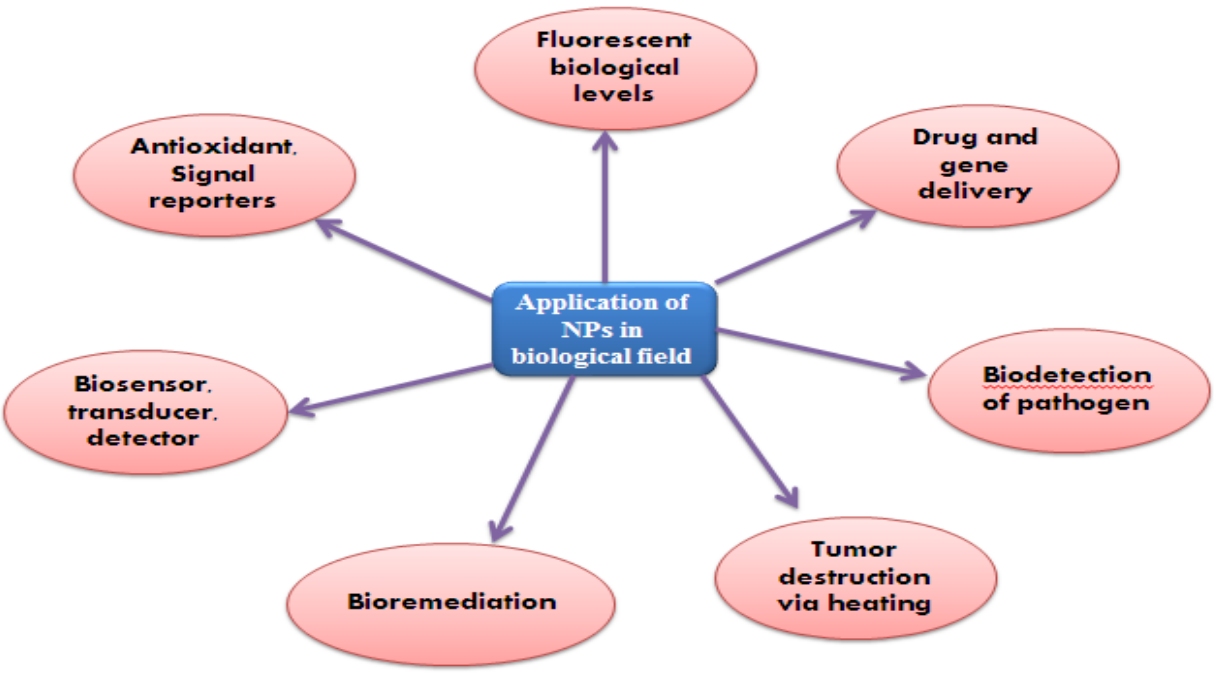


Fig 1: Application of NPs in biological field.

Transition metal oxide NPs such as Cr^{3+} has many applications as catalyst, sensors, superconductors and adsorbents. Metal oxides constitute an important class of materials that are involved in environmental science, biology, chemical sensors, electrochemistry, magnetism and other fields. Chromium exits in different oxidation states and hence many types of oxides of chromium are found, but chromium oxide ($Cr_2O_{3)}$ has attracted much attention due to its application as high temperature resistant material, catalyst, corrosive resistant material and green pigment (Jaswal et al., 2014).Various techniques have been developed to synthesize Cr_2O_3 NPs such as precipitation, precipitation gelation, sol gel, mechano-chemical reaction, and sono-chemical method (Pei et al., 2008). But recently developed biological method using bacteria has the greatest feasibility to be extended to further applications in terms of its easy natural production and low cost. Utilizing chromium resistant bacteria for synthesis of Cr_2O_3 NPs could be an important strategy, which could be used as a sustainable and ecofriendly method of NPs synthesis. Different application of Cr_2O_3 NPs has been given below in Fig 2.

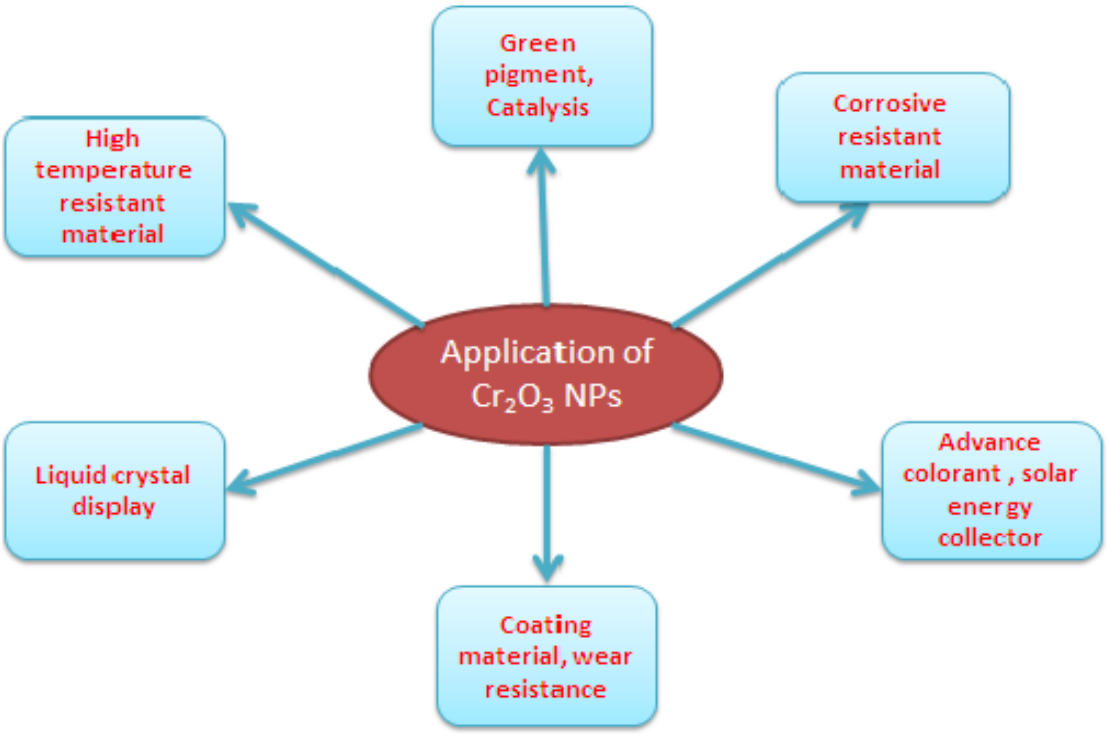


Fig 2: Various applications of Cr_2O_3 NPs in different fields**.**

Review of Literature

The increasing concern with environmental pollution significantly motivates the investigation and development of safe technologies. The group of metals or metalloids having atomic density greater than 4000 kg m^{-3} or 5 times more than water and are natural elements of earth's crust (Hashim et al., 2011). Some of the metals are essential elements in trace amount but when present in excess they possess high level of toxicity.

Heavy metals, because of their non-degradable, persistent and accumulative nature are toxic when present in trace amounts and are source of environmental concern. Toxicity due to trace levels of metals leading to adverse health effect is usually associated with exposure to lead, chromium, cadmium, mercury and arsenic (Kampa et al., 2008). A wide range of industries (mining, metal processing, electroplating and electronics) release such metals into the environment in amounts that can pose great risk to human health. Therefore, metal remediation of wastewater prior to discharge, is of great importance (Gupta et al., 2012). Adsorption is considered as an effective, efficient and economical method for water purification (Gupta et al., 2009). Quality and cost effectiveness of an adsorbent determines the large scale usability of the adsorbent. There has been utilization of different adsorbents such as zeolites (Panuccio et al., 2009), chitosan (Bamgbose et al., 2012), lignocellulose (Shin et al., 2007), activated carbon (Gupta et al., 2012), clay minerals (Hizal et al., 2012) and functionalized polymers (Liang et al., 2010) but large scale application could not be done due to ineffectiveness (due to lack of enough activation sites), high cost, difficulty in separation from waste water or generation of secondary waste. The use of bacteria as biosorbents is a fast growing field in remediation due to their small size, their ubiquity, ability to grow under controlled conditions and resilience to a wide range of environmental situations (Urrutia, 1997). Among different heavy metals that may be removed from aqueous solutions by biosorption, chromium demands special attention as it presents several oxidation states. Cr^{+3} in trace concentration is an essential element in the diet, because it regulates the glucose metabolism in the human body. Excess amounts of Cr^{+6} uptake are very dangerous due to its carcinogenic effect. Cr^{+6} in soils affects the plant growth (Shanker et al., 2003), nonessential for microorganisms and other life forms and when in excess amounts it exerts toxic effect on them after cellular uptake. Cr^{+6} is more toxic than Cr^{+3}, the movement of Cr^{+6} ions and its bioavailability poses a potential threat to the environment. The toxicity of Cr in various industrial effluents is well documented. Cr^{+6} compounds pose health risks to humans,

plants, animals and fishes. Due to its carcinogenicity and mutagenicity, the United States Environment Protection Agency (USEPA) has designated Cr as a "Priority pollutant" or Class A" pollutant (Srinath et al., 2002). Bioremediation of Cr^{+6} has been done by using different groups of microorganisms, but its large scale application is limited due to lack of reusability and separation of the cells from the solution. In addition to this, the problem of limited bio-availability of Cr^{+6} in water leading to lower efficiency. This problem has now been overcome by the emerging field of nanotechnology based bioremediation which played a major role in increasing solubility, bio-availability and subsequently increasing the degradation rate (Mehndiratta et al., 2013)

NPs are having one or more dimensions of the order of 100 nm or less have attracted great attention due to their unusual and fascinating properties, and applications advantageous over their bulk counterparts (Daniel et al., 2004). There are a large number of physical, chemical, biological, and hybrid methods available to synthesize different types of NPs (Liu et al., 2011). Although physical and chemical methods are more popular in the synthesis of NPs, the use of toxic chemicals greatly limits their biomedical applications, in particular in clinical fields. Therefore, development of reliable, nontoxic, and eco-friendly methods for synthesis of NPs is of utmost importance to expand their biomedical applications. One of the options to achieve this goal is to use microorganisms to synthesize NPs. In case of NPs, the ratio of atoms at the material surface is higher than the total number of atoms at its bulk which results in the unusual properties of the nanomaterials such as their catalytic promotion to reactions and their ability to adsorb other materials (Buzea et al., 2007). Thus, NPs have large surface which can carry other substances such as proteins and drugs. NPs are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities (El-Batal et al., 2014). Microbes produce inorganic materials either intra- or extracellularly often in nanoscale dimensions with exquisite morphology. It can be classified into intracellular and extracellular synthesis according to the location where NPs are formed (Simkiss et al., 1989). The intracellular method consists of transporting ions into the microbial cell to form NPs in the presence of enzymes. The extracellular synthesis of NPs involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes (Zhang et al., 2011). NPs have been synthesized by different biological entities such as plants, a number of microorganisms such as bacteria, yeast,

fungi, algae and actinomycetes. Extremophilic actinomycete *Thermomonospora sp*. produces gold NPs extracellularly when exposed to gold ions (Sastry et al., 2003).Yeast *Sandida glabrata* and *Schizosaccharomyces pombe* were used for synthesis of cadmium sulphide (CdS) nanocrystals using cadmium salts which have been used in quantum semiconductor crystallites (Dameron et al., 1989). NPs with well-defined dimension and monodisperity has been obtained using fungi ; such as fungus *Verticillium sp*. has been used for formation of gold NPs with fairly well defined dimension and good mono-dispersity (Mukherjee et al., 2001). Plants have been observed to be a good source of production of quantum dots which have a wide application in nanobiotechnology; synthesis of quantum dots has been observed by a living plant alfalfa (Torresdey et al., 2003).

It is widely accepted that microorganisms that can tolerate heavy metal stress may become potential bio factories for the synthesis of metal NPs (Iravani et al., 2014). These microorganisms survive and grow in high metal concentration due to several mechanisms like efflux ECM system, bioaccumulation, biosorption, alteration of solubility and toxicity via reduction or oxidation, precipitation of metals and lack of specific metal transport systems (Husseiny et al., 2007). In bacteria most metal ions are toxic to them; bacteria tolerate them by the defense mechanism developed like the reduction of ions and formation of water insoluble complex (Sastry et al., 2003), thus NPs is a byproduct of defensive mechanism against a metal stress (Pantidos et al., 2014). Many microorganisms are also known for production of metallic NPs and nanostructured mineral crystals having control over their size, shape and composition having properties similar to chemically synthesized NPs (Li et al., 2011). Examples include production of silver NPs by *pseudomonas stutzeri*, formation of palladium NPs using sulphate reducing bacteria (Gericke et al., 2006). Utilization of microorganisms for synthesis of NPs with different size, shape, controlled monodispersity can be a novel biological method of nanoremediation of toxic metals (Cameotra et al., 2010). There has been a considerable progress in the synthesis of NPs in the past decades .Transition metal oxide NPs plays an important role in many areas of chemistry, physics and material science. Metal oxide NPs exhibits unique chemical properties due to their limited size and high density of corner or edge surface sites (Gareia et al., 2004). Among metal oxides chromia (Cr_2O_3) plays an important role as coating material, wear resistance, heterogonous catalyst (Rao et al., 2009), advanced colorant, pigment and solar energy collector. A number of physicochemical techniques has been utilized for the

synthesis of Cr_2O_3NPs, such as sol gel process using hydrazine monohydrate ($(NH_2)_2.H_2O$) (Balouria et al., 2012), precipitation method utilizing ammonia as precipitating agent (Jaswal et al., 2014) and solvent free microwave irradiation technique by reacting $CrCl_3.6H_2O$ and NH_2-CO-NH_2 (Meenambika et al., 2014). Apart from chemical methods, green synthesis of NPs has also been done by the reduction of chromium salt solution by biological systems. Such as ectomycorrhizal fungi (Sawrnakar et al., 2009), *Tridax procumbens* leaf extract which contain carbohydrate as a major reducing agent (Ramesh et al., 2012), *Mukia Maderaspatana* plant (Rakesh et al., 2013) and bacteria *Bacillus Subtilis* (Annamalai et al., 2014) have been reported for successful synthesis of Cr_2O_3 NPs and recovery of heavy metal. The utilization of biological method offers environment friendly and cheapest method hence attracting scientists to synthesize NPs by using biological method (Ananda et al., 2013). Advantage of biological method of NP synthesis over physical/chemical method has been represented below in Fig 3.

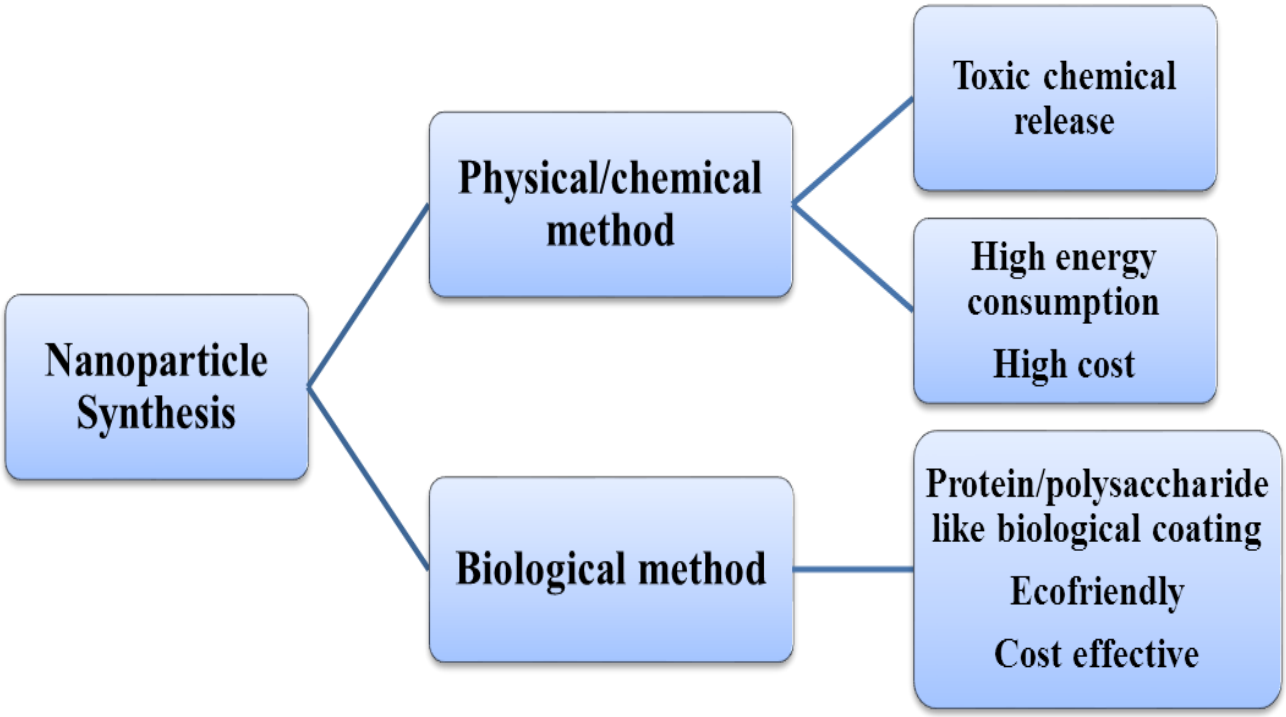


Fig 3. Advantages of biosynthesis over conventional methods (Nirmala et al., 2013)

Objectives

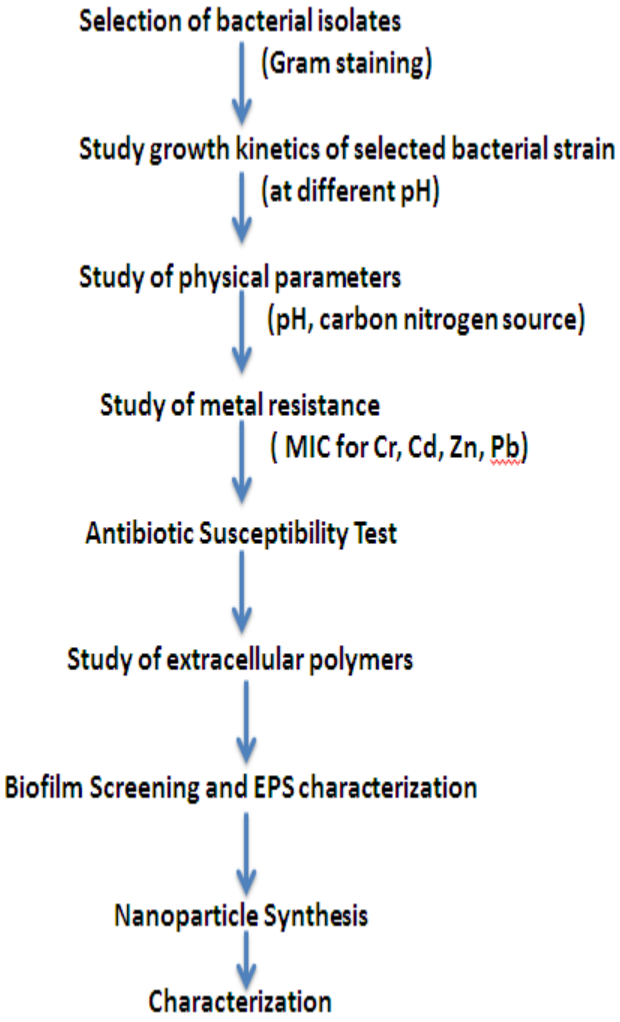
1. Optimization of growth parameters and characterization of acidotolerant *Lysinibacillus* sp. RTA 01

2. Characterization of biofilm-EPS extracted from bacterium *Lysinibacillus* sp. RTA 01

3. Adsorption study of Cr^{6+} metal ions by the biofilm- EPS formed by *Lysinibacillus* sp. RTA 01

4. Biosynthesis and characterization of chromium oxide NPs

Work Plan



Materials and Methods

4.1 Isolation of bacterial isolates

Isolation and screening of bacterial isolates from acidic rubber effluent discharge have been carried out on the basis of their tolerance to metal concentration and growth conditions. Minimum inhibitory concentration (MIC) against Cr^{+6} was determined in terms of $K_2Cr_2O_7$ for the isolates by micro-broth dilution technique, and the bacterial isolate *Lysinibacillus* sp. RTA 01 (accession number: KP986590) showing highest level of resistance towards Cr^{+6} was selected for further study.

4.2 Gram staining of *Lysinibacillus* sp. RTA 01

Gram staining of bacterial isolates was performed to differentiate Gram positive and Gram negative depending upon their chemical and structural make up of cell-wall (Claus et al., 1992).

4.3 Growth curve of *Lysinibacillus* sp. RTA 01

Overnight grown culture of *Lysinibacillus* sp. RTA 01 in Luria Bertani (LB) broth was used as an inoculum for the analysis of growth pattern. It was further inoculated into LB medium with different pH of 4, 5, 6 and 7 and incubated at 37^0C temperature with continuous shaking at 120 rpm. Growth pattern was observed by measuring the optical density at 595 nm at an interval of 1 h in microtiter plate reader (Victor X3 2030 multilabel reader, Perkin Elmer, USA).

4.4 Optimization of carbohydrate and nitrogen sources

Bushnell Hass Broth (BHB) media supplemented with 125 ppm Cr^{+6} solutions and with different carbohydrate (glucose, fructose, sucrose, and galactose) and nitrogen (yeast extract, potassium nitrate, urea, beef extract) sources were taken in 96 well plate. Overnight grown culture of *Lysinibacillus* sp. RTA 01 was inoculated with BHB media with culture as positive control and media with different carbohydrate and nitrogen source as negative control. The growth of *Lysinibacillus* sp. RTA 01 was optimized after staining it with crystal violet and then washing in ethanol and measured the optical density at 595 nm after 24 h in terms of destained ethanol in microtiter plate reader (Victor X3 2030 multilabel reader, Perkin Elmer, USA).

4.5 MIC (Minimum inhibitory concentration)

MIC is the lowest concentration which inhibits the visible growth of microorganisms after overnight incubation. Different methods were used to determine the MIC of isolates.

4.5.1. *Well diffusion method*

Lysinibacillus sp. RTA 01 was cultured in LB culture plates with different concentrations of Cr^{+6} solution ranging from 125 to 1000 ppm in wells. After, overnight day incubation at 37˚C, zone of inhibition was measured manually (Islam et al., 2008).

4.5.2. *96 well plate method*

Different concentration of Cr^{+6} solutions along with other heavy metal solutions (Pb^{2+} Zn^{2+} & Cd^{2+}) in LB broth were taken in 96 well plate. Respective wells were inoculated with 20 µl of overnight grown *Lysinibacillus* sp. RTA 01 culture and incubated at 37^0C for 24 h. After, 24 h, the absorbance of respective wells were taken at 595 nm in microtiter plate reader (Victor X3 2030 multilabel reader, Perkin Elmer, USA) and compared with positive control without any metal supplement (Mangwani et al., 2014).

4.6. Antibiotic susceptibility test

The antibiotic susceptibility test of *Lysinibacillus* sp. RTA 01 against different antibiotics like norfloxin, vancomycin, azithromycin, neomycin and tetracycline of different group was performed by Kirby Bauer's disk diffusion method using commercially available kits (Bauer et al., 1996).

4.7. Biofilm screening and growth studies

20 µl *Lysinibacillus* sp. RTA 01 was transferred to microtiter plate with 180 µl of media supplemented with ($K_2Cr_2O_7$) solutions. Media without metal was taken as control. At an interval of 24h, 48h, 72h and 96h the media was taken out, washed twice air dried an stained with 0.2% crystal violet. Destained with ethanol and optical density was measured at 595 nm in microtiter plate reader (Victor X3 2030 multilabel reader, Perkin Elmer, USA) in terms of destained ethanol (Mangwani et al., 2014).

4.8. Fluorescence imaging of biofilm formation in presence of Cr^{6+} metal solution

Overnight grown culture of *Lysinibacillus* sp. RTA 01 was inoculated in LB media supplemented with Cr^{6+} solutions and media without metal supplemented was taken as control. Sterile glass slide were added in each tube. Slides in duplicates were taken out in 24 h interval and then washed with PBS.

It was then stained with acridine dye for 5 min and again washed with PBS and thin cover slip was placed over the biofilms. The slides were observed in fluorescence microscope (Olympus,

IX71, Japan) to obtain architecture of biofilm growth and the integrated density of biofilm (Chakraborty et al., 2014).

4.9 Extraction of EPS (Extracellular polymeric substance) from *Lysinibacillus* sp. RTA 01

Overnight grown culture of *Lysinibacillus* sp. RTA 01 was centrifuged at 6900 rpm for 15 min and separated cell pellet was then inoculated in Minimal Davis broth supplemented with 1% glucose solution. Cells after grown up to stationary phase were centrifuged and to the collected supernatant added equal volume of chilled ethanol (99.9%) and incubated overnight at 4^0 C for precipitation of EPS. After overnight incubation it was then centrifuged and the pellets obtained were dried in desiccator and lyophilizer (Gong et al., 2009).

4.10 Chromium reduction assay

The amount of chromium adsorbed by bacteria was determined by 1, 5- diphenyl carbazide (DPC) assay to determine the Cr^{6+} present in the solution (Focardi et al., 1992). R2A media supplemented with 50 ppm Cr^{6+} solution was inoculated with *Lysinibacillus* sp. RTA 01 and allowed to grow overnight at 120 rpm, 37^0C for 48 h. Supernatant was collected after centrifugation at 6900 rpm for 15 minutes. 20 µl/ml of DPC solution was added adjusted pH around 2±0.5 and reading in UV Visible Spectrophotometer was taken at 540 nm and then compared with the standard curve to estimate the amount of Cr^{6+} adsorbed by bacterial cells.

4.11. Biosynthesis of nanoparticles

NPs were synthesized from bacterial cell mass (pellet), cell free media (supernatant) and EPS using different concentration of Cr^{6+} at different time interval was presented in Table 2.

4.11.1. Preparation of $K_2Cr_2O_7$ solution

29.4 g of $K_2Cr_2O_7$ was dissolved in 100 ml of distilled water and stirred for 15 min. An orange color $K_2Cr_2O_7$ solution of 1M obtained. From this 0.01M, & 0.001 M Cr solution was obtained by serial dilution method.

4.11.2. Collection of supernatant and pellet

Overnight grown culture of *Lysinibacillus* sp. RTA 01 was taken and centrifuged at 6900 rpm for 15 min. The separated pellets were kept in desiccator and the collected supernatant and pellet were used for synthesis of NPs.

4.11.3. NP synthesis from bacterial cell supernatant

In previously collected supernatant of *Lysinibacillus* sp. RTA 01 different concentration of (0.01M & 0.001M) Cr^{6+} ($K_2Cr_2O_7$) was added drop wise and allowed to incubate at 37˚C with continuous shaking condition. Samples were taken out at an interval of 24h, 48h, and 72h with significant color change (orange to yellowish green) in solution. The collected samples were centrifuged at 7200 rpm for 15 min to obtain pellet and supernatant for further characterization.

4.11.4. NPs synthesis from bacterial cell mass

Cell mass of *Lysinibacillus* sp. RTA 01 was taken and a stock solution 0.1g/L was prepared by dissolving in PBS. Different concentration (0.01M & 0.001M) of Cr^{6+} ($K_2Cr_2O_7$) was added drop wise with continuous stirring and incubated at 37^0 C with 120 rpm. Samples were taken out at an interval of 24h, 48h, and 72h with significant color change (orange to yellowish green) in solution. The samples were then centrifuged at 7200 rpm for 15 min to obtain pellets. After washing twice with Milli Q water, obtained pellet was dissolved in Milli Q water and supernatant collected were taken for characterization.

Table 2. Different concentration of Cr^{+6} used for synthesis of NPs along the time of incubation

Sample description	Concentration of Cr^{+6}	Incubation time
Cell pellet	0.001M Cr	24 h
	0.01M Cr	24 h
	0.01M Cr	48 h
	0.01M Cr	72 h
Supernatant	0.001M Cr	48 h
	0.01M Cr	24 h
	0.01M Cr	48 h
	0.01M Cr	72 h
EPS	0.001M Cr	48 h
	0.01M Cr	48 h

4.11.5. NPs synthesis from EPS

Extracted EPS from *Lysinibacillus* sp. RTA 01 was taken and dissolved in PBS to achieve a solution 1mg/10 ml concentration. Different concentration of Cr^{6+} ($K_2Cr_2O_7$) was added drop wise with continuous stirring by magnetic stirrer (500 rpm). After 48h with

significant color change (orange to yellowish green) stirring samples were centrifuged and pellets and supernatant were collected for further characterization.

4.12. Characterization of synthesized Cr^{+3} NPs

4.12.1. UV Visible Spectroscopic Analysis

The absorption spectra of NPs synthesized from above mentioned methods were obtained by using Double Beam Scanning Visible Spectrophotometer, before scanning the solid sample were dissolved in autoclaved distilled water and samples were sonicated for 15 minutes in sonicator (Sartorius stedim, Labosonic M, Germany). 3 ml of samples were taken in cuvette and scanned in the range 200 to 700 nm.

4.12.2. XRD Analysis

X-ray diffraction was done by using Rigaku Miniflex X ray diffractometer (Japan). The dried solid synthesized NPs samples were scanned in the range 20^0 to 60^0 with scanning rate 10^0 at angle of diffraction 2 θ degrees to analyze the size and crystallinity of the prepared NPs.

4.12.3. FTIR Analysis

FTIR spectra of the synthesized NPs were taken to determine the functional groups present in the synthesized NPs and compared with the control to see the addition or deletion of groups. For FTIR analysis NPs were characterized by ATR-FTIR (Bruker, Germany) in the range 4000 - 500 cm^{-1}.

4.12.4. Zeta potential Analysis

Zeta potential of the synthesized NPs were performed by Nano Zeta sizer (Nano series ZS90, Malvern instruments Ltd., UK) to determine the potential difference between the dispersed medium and the stationary layer of fluid attached to it and hence to determine charge and stability of the synthesized NPs. Solid samples were dissolved in autoclaved Milli Q water, both solid and liquid samples were sonicated for 15 min before analysis.

4.12.5. Electron Microscopy

20 µl of above mentioned synthesized NPs suspension were taken on grease free glass slide by drop cast method. Characterization of NPs was performed by (Nova Nano SEM 450/FEI, USA) equipped with energy dispersive X-ray spectrometer (Hitachi, Japan) at different magnifications to study the size and shape of synthesized NPs along with elemental analysis.

RESULTS

Gram staining

Under light microscope, *Lysinibacillus* sp. RTA 01 found to be gram-positive and rod shaped (Fig 4).

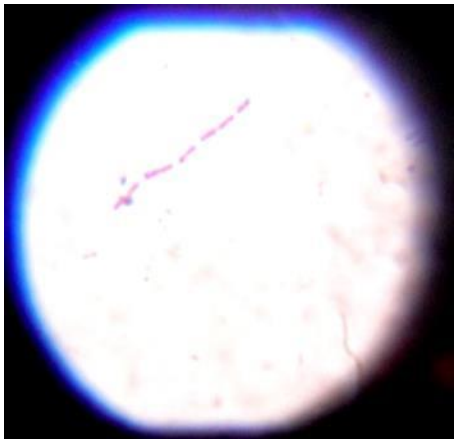


Fig 4. Gram Staining of *Lysinibacillus* sp. RTA 01

Growth Curve

The bacterial isolate *Lysinibacillus* sp. RTA 01 was allowed to grow in different pH (4, 5, 6 and 7) and then the growth was measured by taking absorbance in microtiter plate reader (Victor X3 2030 multilabel reader, Perkin Elmer, USA) at 1 h interval for 24 h. The growth curve of *Lysinibacillus* sp. RTA 01 is presented below in Fig 5.

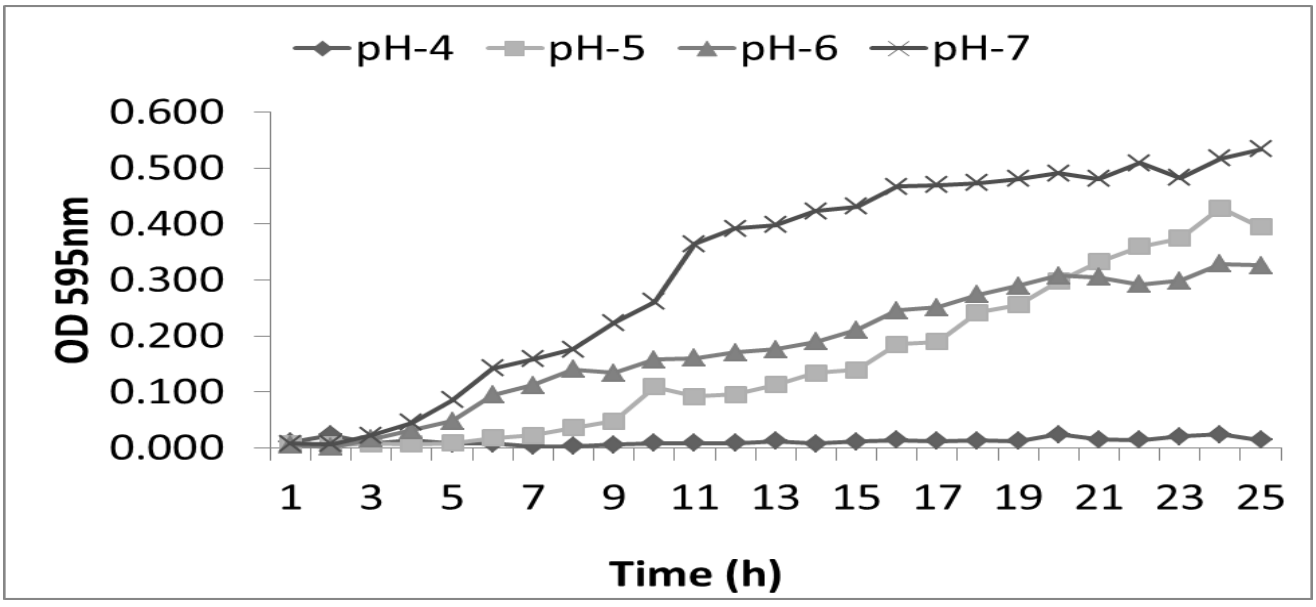


Fig 5. 24 h growth curve of *Lysinibacillus* sp. RTA 01 at different pH

It was observed that bacterial isolate *Lysinibacillus* sp. RTA 01 shows optimum growth at pH 7 and there is significant growth at pH 5 and 6 indicating it could tolerate moderate acidic environment.

Optimization of carbohydrate and nitrogen sources

Lysinibacillus sp. RTA 01 is found to have optimum growth in glucose as carbon source compared to other carbon sources; the results are shown in graphical manner below in Fig 6.

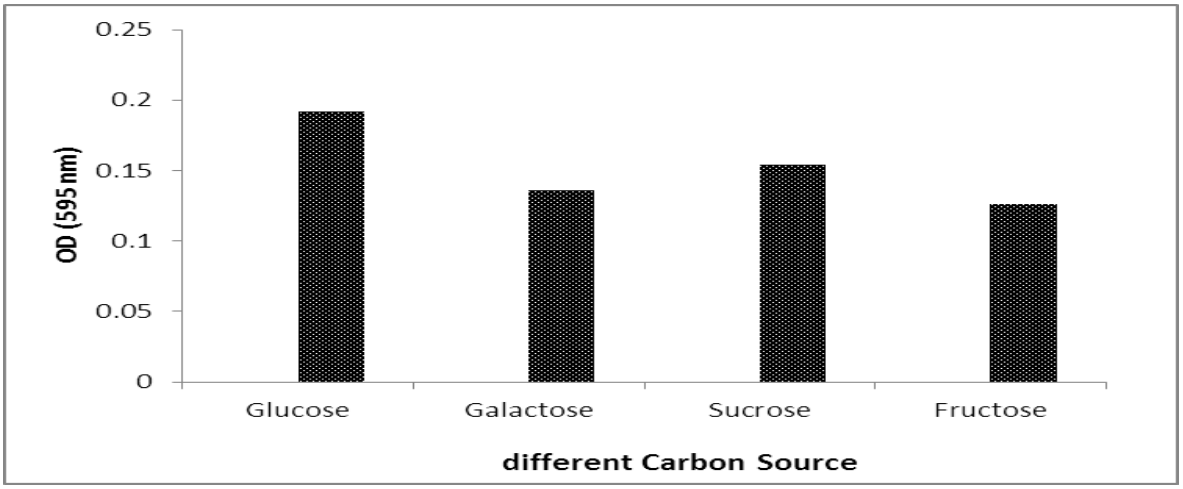


Fig 6. Growth of *Lysinibacillus* sp. RTA 01 in different Carbon source

The growth of *Lysinibacillus* sp. RTA 01 in different nitrogen sources is given below in Fig 7. It was observed that there is optimum growth of the isolate in beef extract as nitrogen source compared to others.

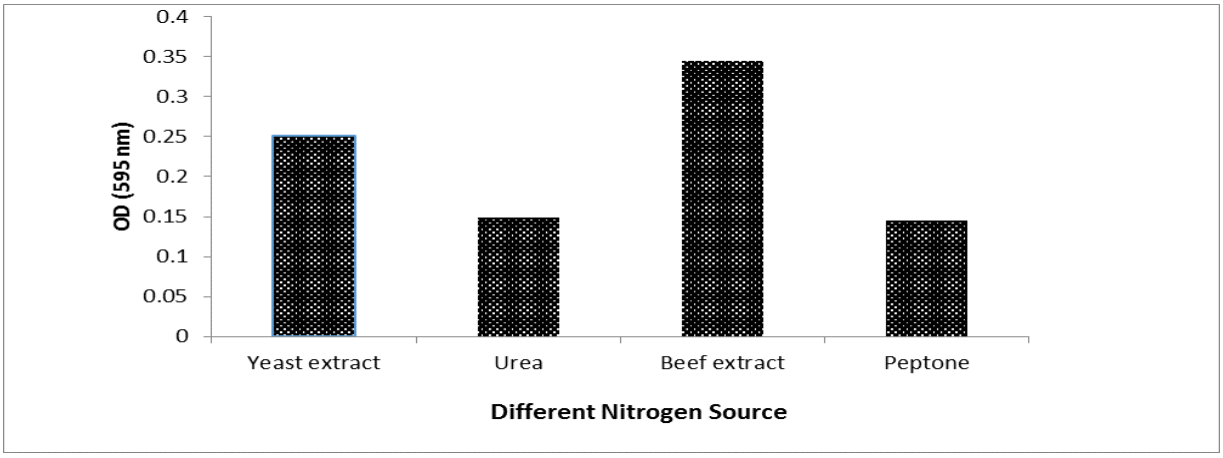


Fig 7. Growth of *Lysinibacillus* sp. RTA 01 in different Nitrogen source

Minimum inhibitory concentration

MIC of *Lysinibacillus* sp. RTA 01 was determined by various methods such as well method and 96 well plate assays against different metals to determine the tolerance level of *Lysinibacillus* sp. RTA 01 against different metals. The MIC value of *Lysinibacillus* sp. RTA 01 is shown below in Fig 8 in graphical form.

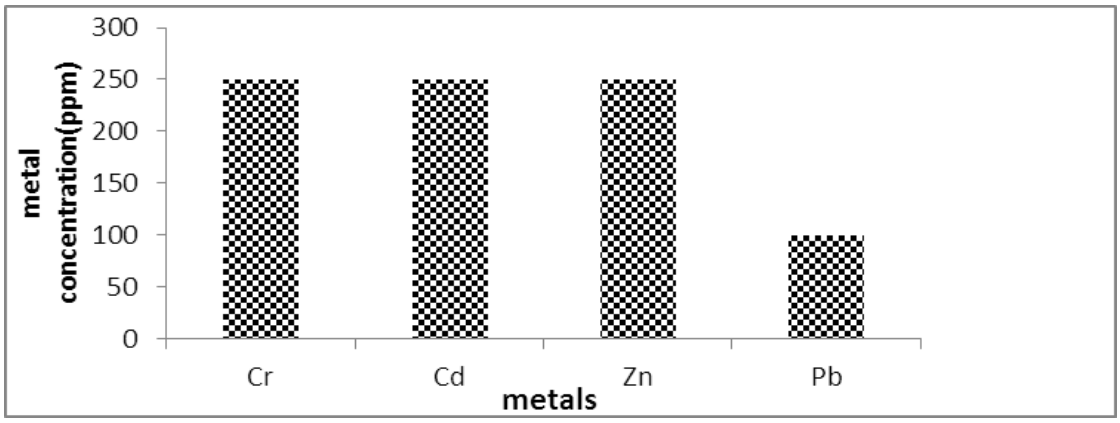


Fig 8. MIC of *Lysinibacillus* sp. RTA 01 against different metals

In micro titer plate method MIC of *Lysinibacillus* sp. RTA 01 against Cr, Cd and Pb metal ($K_2Cr_2O_7$, $(CdNO_3)_2$ and (Pb $(NO_3)_2$ as Cr, Cd and Pb source respectively) was found to be 250 ppm. However, it was 100 ppm against Zn where $ZnSO_4$ solution was used as Zn source.

Antibiotic susceptibility test

Antibiotic susceptibility test of *Lysinibacillus* sp. RTA 01 was done against different groups of antibiotic such as norfloxin, vancomycin, azithromycin, neomycin and tetracycline. The diameter of zone of inhibition was measured and then compared with the chart to determine the degree of susceptibility. Fig 9 shows the zone of inhibition of *Lysinibacillus* sp. RTA 01 against different antibiotics.

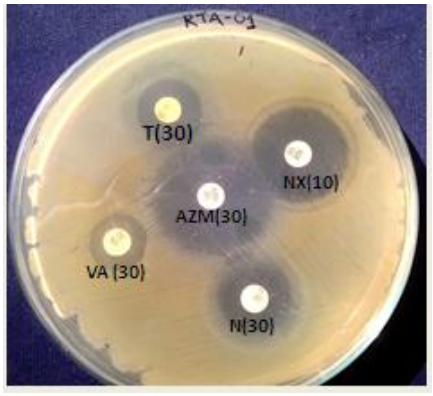


Fig 9. Antibiotic susceptibility test of *Lysinibacillus* sp. RTA 01 showing zone of inhibition

The diameter of the zone of inhibition and degree of susceptibility of *Lysinibacillus* sp. RTA 01 against the antibiotics was represented in tabular form in Table 3 given below.

Table 3. Antibiotic Susceptibility of *Lysinibacillus* sp. RTA 01 against the antibiotics (norfloxin(NX) , vancomycin(VA), azithromycin(AZM), neomycin(N) and tetracycline(T).

Antibiotics	*Lysinibacillus* sp. RTA 01
N_{30}	1.5cm (resistance)
VA_{30}	1.2 cm (resistance)
AZM_{30}	2.8 cm (susceptible)
NX_{10}	2.2 cm (susceptible)
T_{30}	1.4 cm (resistance)

It was found that *Lysinibacillus* sp. RTA 01 strain was resistance to vancomycin, azithromycin, neomycin and tetracycline and susceptible to norfloxin.

Biofilm assay

The biofilm assay of *Lysinibacillus* sp. RTA 01 in presence of different Cr^{6+} and control (without metal) shown below in Fig 10 It was observed that in absence of metal the biofilm growth declines at 48 h but in presence of Cr^{6+} there was decline in biofilm growth at 96h.

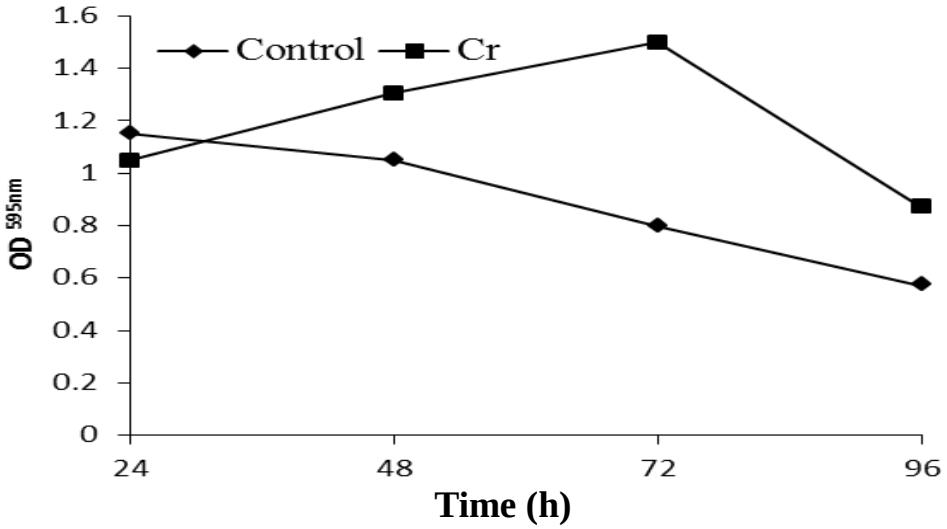


Fig 10. Microtiter plate Assay of biofilm formation of *Lysinibacillus* sp. RTA 01 in presence of toxic metal (Cr^{6+}) compared with control (without metal)

Further characterization of biofilm formation was done by fluorescence microscopy and observed the architecture of biofilm formation in presence of Cr^{6+} supplemented media .The images of fluorescence microscope it was observed under fluorescence microscope at different time interval (24h, 48h, 72h and 96h) shown below in Fig 11 from which it can be observed that there is gowth of biofilm in 24 h , it increases in 48 h and 72 h after which it starts dirupting showing less growth at 96h.

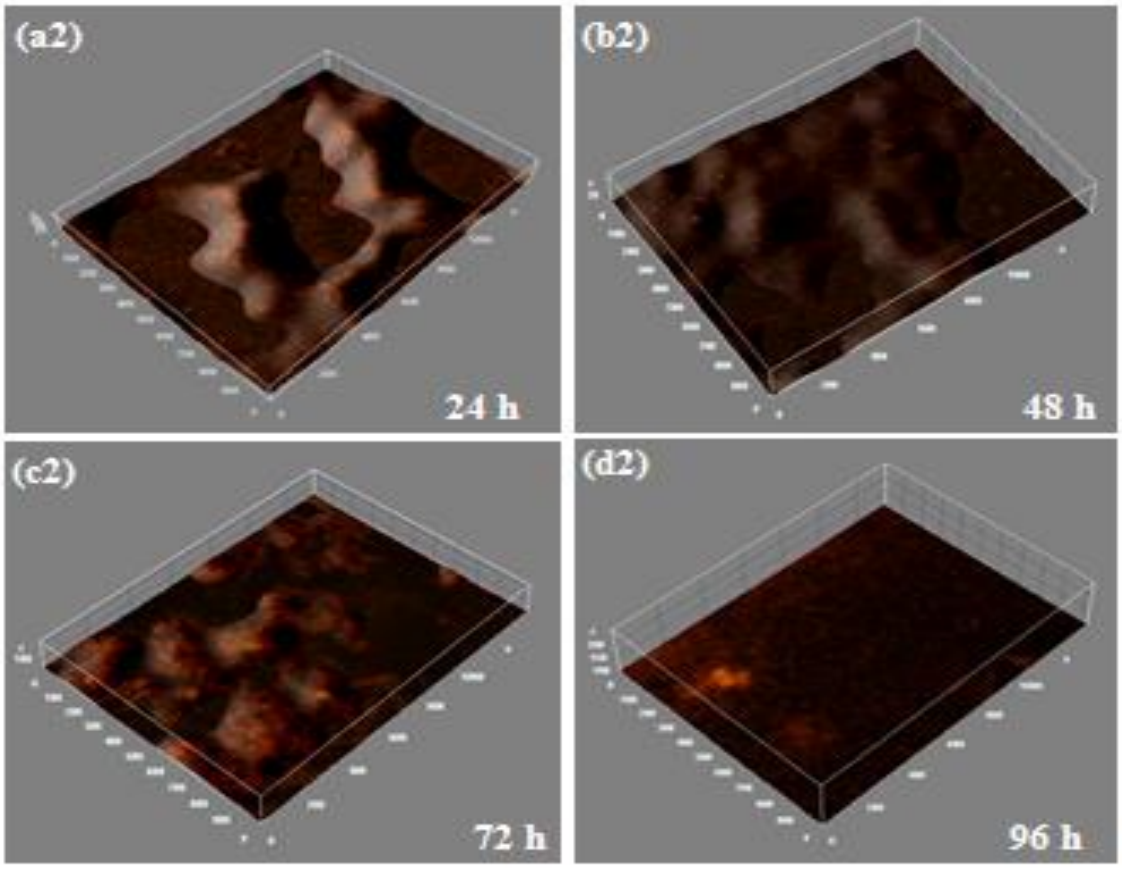


Fig 11. Stages of biofilm formation in presence of Cr^{6+} of *Lysinibacillus* sp. RTA 01 after 24 h, 48 h, 72 h & 96h as observed under fluorescence microscope

FTIR analysis

The EPS extracted at different concentration of Cr^{6+} (10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm) were analyzed by ATR- FTIR. The spectrum is represented below in Fig 12.

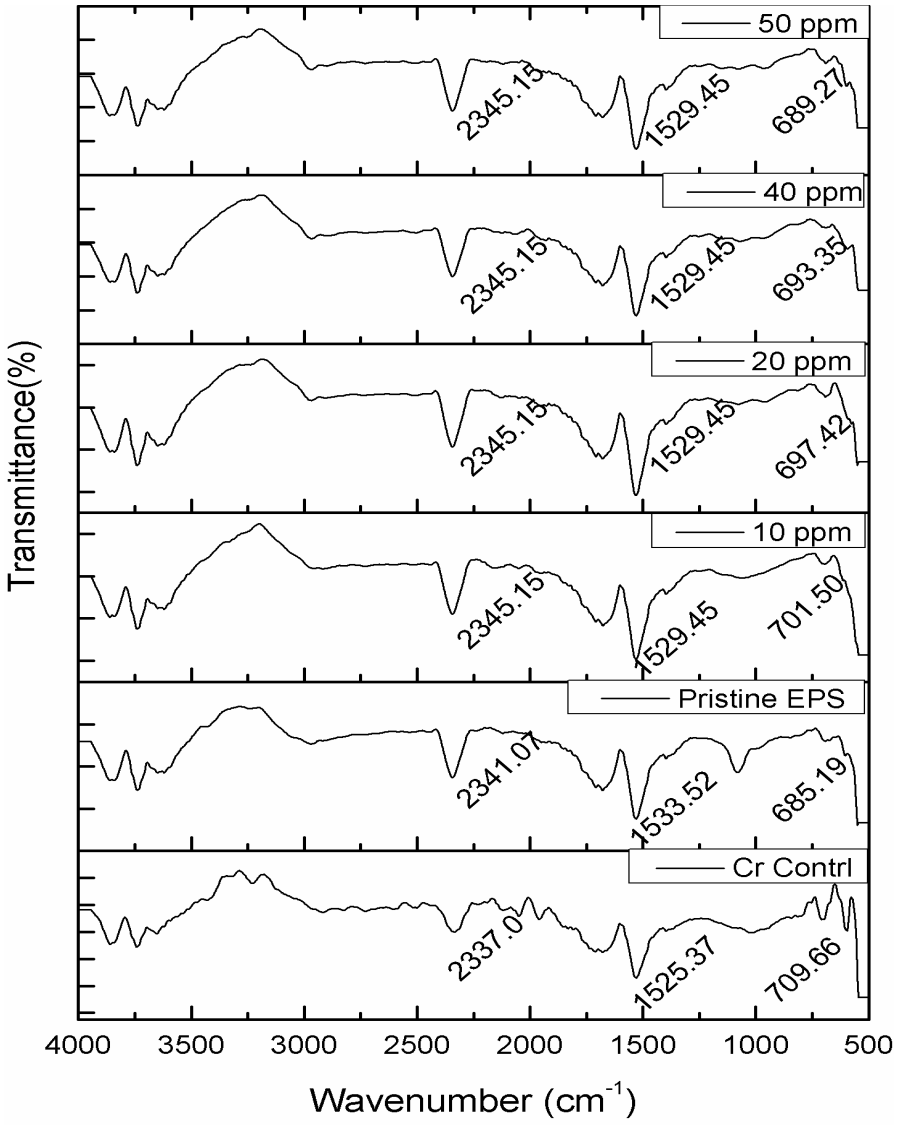


Fig 12. FTIR spectra of EPS at different concentration of Cr^{6+}

There was presence of overlapping peaks at 2337, 1529 and variable peaks that is 701.5 cm^{-1} at 10 ppm Cr^{6+} and there was decrease in value observed as the concentration of Cr increases. The peaks found 2337 to 2345.15 cm^{-1} correspond to-S-H – stretch, peaks found at 1533.52 cm^{-1} in case of control (Cr^{6+}solution) peak shift found when EPS interacts with the Cr^{6+} corresponding to –N-O stretch. The peak at 685.19 cm^{-1} found in pristine EPS and Cr^{6+} the peak obtained at

709.66 cm^{-1}. In presence of different concentration of $Cr^{6+,}$ EPS showed variable peaks in between 709.66 and 685.19 which indicates metal interaction with functional groups of EPS.

Standard curve of Cr^{6+}

Standard curve of chromium was done by Diphenyl Carbazide Assay. Absorbance was taken in UV Visible spectrophotometer at 540 nm and plotted (Fig 13). From this standard curve, adsorption of Cr^{+6} by bacterial cell has been calculated.

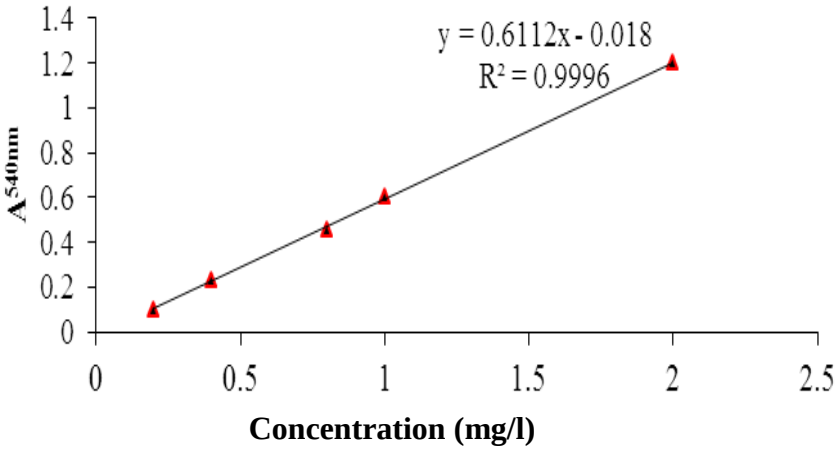


Fig 13. Standard curve of Cr^{6+}

Adsorption of Chromium by bacterial Cell

The percentage of removal Cr^{6+} by *Lysinibacillus* sp. RTA 01 after 48 h of incubation with 50 ppm Cr^{6+} supplemented media was found to be 53.94% compared control (without bacteria).

Cr^{6+} reduction assay

In DPC assay the 1, 5- diphenyl carbazide combines with Cr^{6+} present in the solution resulting in the formation of a colored complex which was then measured by UV-Visible Spectrophotometer. Then the absorbance was compared with the control to determine the amount of Cr adsorbed by bacterial cell. From the adsorption study it was found that *Lysinibacillus* sp. RTA 01 reduces 53.94% of Cr^{6+} from the aqueous solution. From color change, it could be easily observed that control having more amount of Cr^{6+} (bright pink coloration) and as the amount of Cr^{6+} decreases the coloration becomes fainter indicating reduction of Cr^{6+} to Cr^{3+} by the bacterial cell (Fig 14).

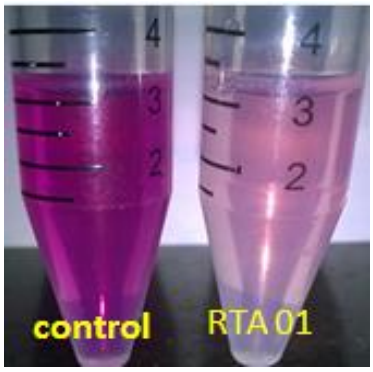


Fig 14. DPC assay of *Lysinibacillus* sp. RTA 01 compared with the control, as the concentration of Cr^{6+} decreases the color becomes fainter

Lysinibacillus sp. RTA 01 was found to have capacity to remove of 53.94% of Cr^{6+} from aqueous solution (Table 4).

Table 4. Cr^{6+} adsorption by *Lysinibacillus* sp. RTA 01

STRAIN	ABSORBANCE	Cr REMOVAL (%)
Lysinibacillus sp. RTA 01	0.899	53.94
CONTROL	1.952	0.0

Synthesis of NPs

Nanoparticles synthesized from bacterial pellet, supernatant, EPS, culture with different concentration of Cr^{6+} (0.1 and 0.001 M) which could be observed from the color change from orange to yellowish green color (Fig 15).

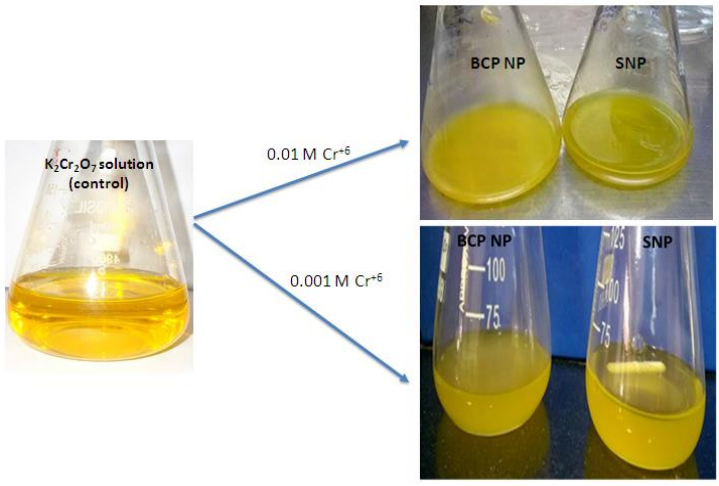


Fig 15. NPs synthesized from pellet and supernatant at different concentration of Cr^{6+} (Color change from yellowish orange to yellowish green indicates formation of NPs)

Characterization of NPs

UV Visible spectra, FTIR, DLS, Zeta, FESEM, EDX, and XRD of samples were done for different characterization of synthesized NPs.

UV Visible Absorption Spectra

NPs were synthesized from 0.001 M Cr shows sharp peaks in 368 and 386 in SNP & Cr control and in BCS NP peaks were found at 367 & 385 (Fig 16).These peaks indicate the formation of Cr^{3+} NPs (Ananda et al., 2013).

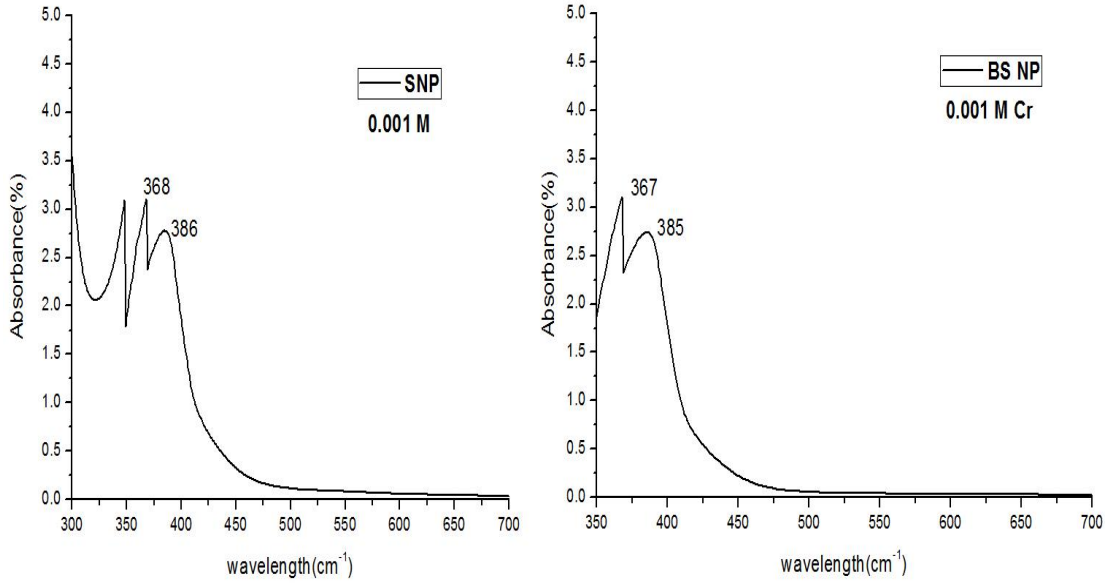


Fig 16. UV absorption spectra of a) BS NP (bacterial cell supernatant NP) b) SNP (Supernatant NP) at 0.001 M Cr^{6+} concentration

The UV Visible spectra of NPs synthesized from 0.01M Cr with EPS, cell pellet and supernatant shows characteristic peaks at 422, 416, 419, 445 nm for BS NP 48 h, BS NP 24 h , EPS NP and SNP respectively as shown in Fig 17. From the literature review it was found that these peaks reveal the formation of Cr^{3+} NPs.

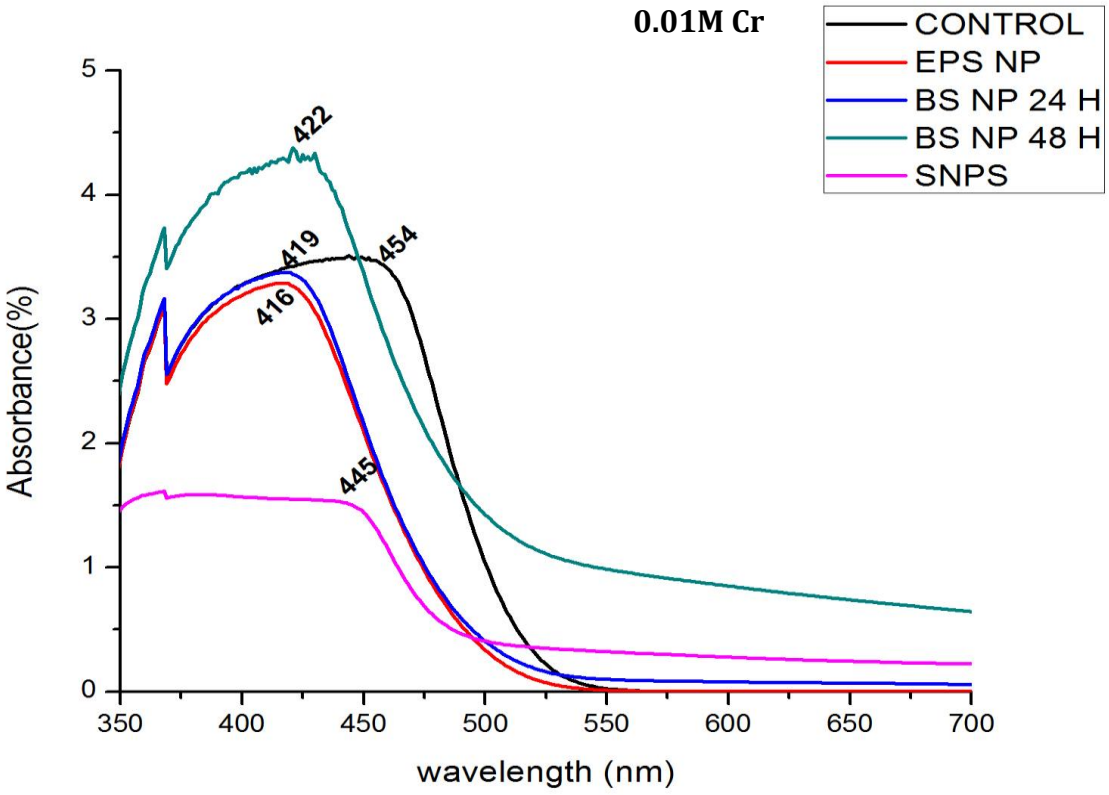


Fig 17. UV absorption spectra of EPS NP, BS NP (bacterial cell supernatant NP) 24, 48 h, SNP (supernatant NP), at 0.01 M Cr^{6+}

NPs synthesized from 0.01M Cr and *Lysinibacillus* sp. RTA 01 Culture ,UV Visible spectra of the supernatant and pellets shows characteristic peaks at 361, 362 and 363 nm in BCP NP 24, 48 and 72 h respectively indicating the formation of Cr^{3+} NPs.

XRD spectra

XRD spectra were taken in Rigaku Miniflex X ray diffractometer Japan. A number of peaks were found in bacterial cell pellet (control) such as 21.3, 24.45, 25.85, 28.25, 33.45, 40.7, 45.65, 50.5˚ as shown below in Fig 18. (a) compared to bacterial cell pellet NP (BCP NP) only two peaks are observed at 24.2 and 28.25 indicating the presence of Cr_2O_3 NP (Fig.18 (c)). In EPS (control) there was also presence of number of peaks 21.3, 28.85, 27.05, 30.55, 32.15 and 33.2 as shown in Fig 18 (b). and in case of NPs synthesized from bacterial supernatant and 0.01M Cr and the pellets obtained from them (SNP P) shows characteristic peaks at 23.05 and 28.35 indicating the presence of Cr_2O_3 NPs (Fig 18 d).

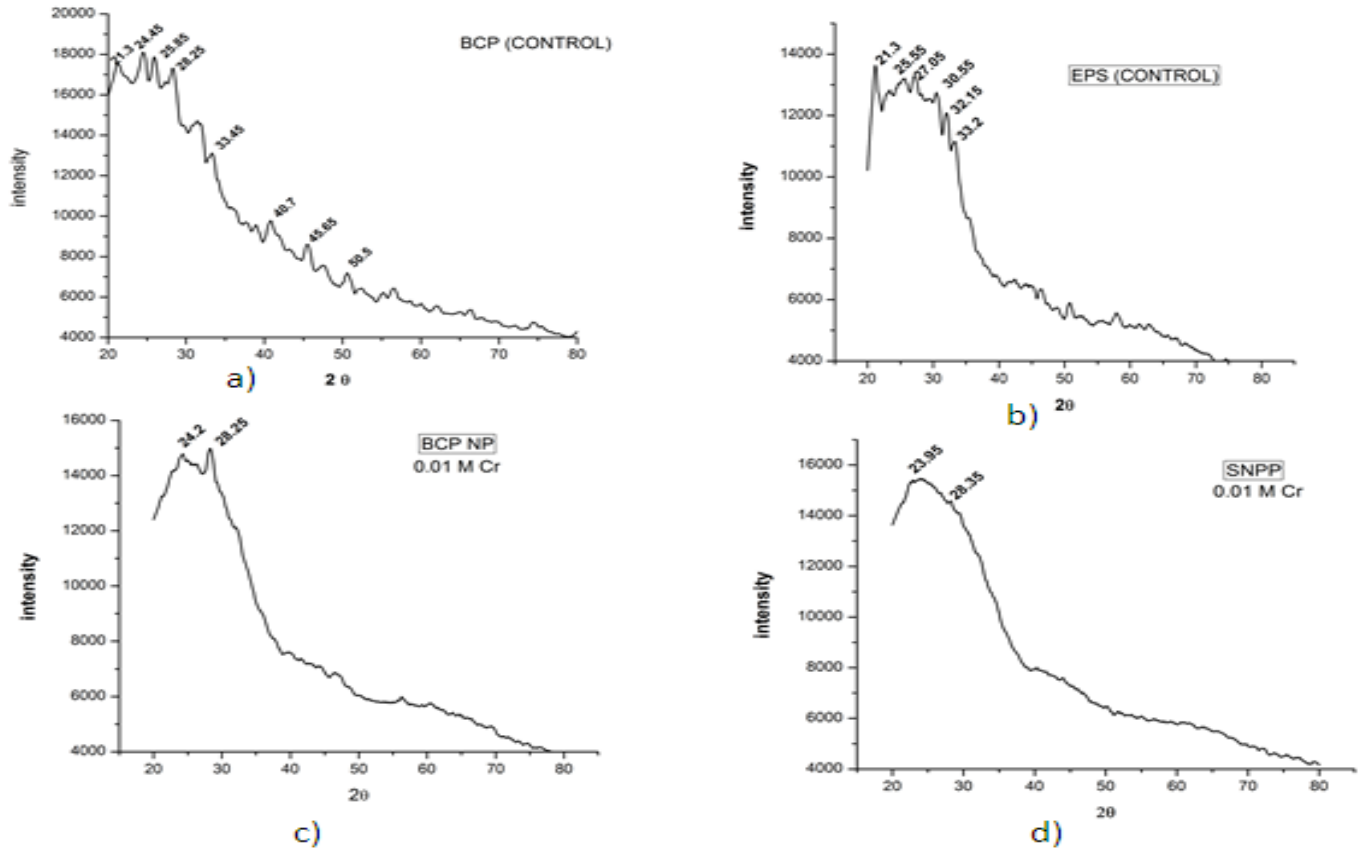


Fig 18. XRD peaks of a) BCP(Bacterial Cell Pellet) Control, b) EPS(control), c) BCP NP(Bacterial Cell Pellet NP), d) SNPP(Supernatant NP pellet).

Particle size calculation by Scherrer equation: $B(2\theta) = \dfrac{K\lambda}{L\cos\theta}$

K is a dimensionless shape factor (0.94),
λ is the X-ray wavelength(1.54 nm)
B is the angular line width of half maximum.
θ is the Bragg angle.
L crystalline size

From the above mention equation, crystallite size of BCP SNPP NPs was found to be 1-3 nm and 2-5 nm respectively.

FTIR spectra of NPs

FTIR spectra of samples were taken for detection of functional groups present in the synthesized NPs. The synthesized NPs from 0.001 M Cr showed characteristic peaks at 2337 cm^{-1} in Cr^{6+} solution (control) and there was shift in peak value in EPS NP, BCS NP and S NP .Peaks at 1533, 1529 cm^{-1} were due to CH_2 stretch and N-H stretch were found in bacterial supernatant NP and Bacterial cell supernatant NP. Peaks were found at 689 cm^{-1} which confirms the formation of Cr_2O_3 in BCS NP and BS NP. (Fig 19).Metal oxide Cr_2O_3 generally absorbs below 1000 cm^{-1} due to its interatomic vibrations. Peaks at 689 in S NPs and 697 cm^{-1} in BCS NPs confirm the presence of crystalline Cr_2O_3 NP.

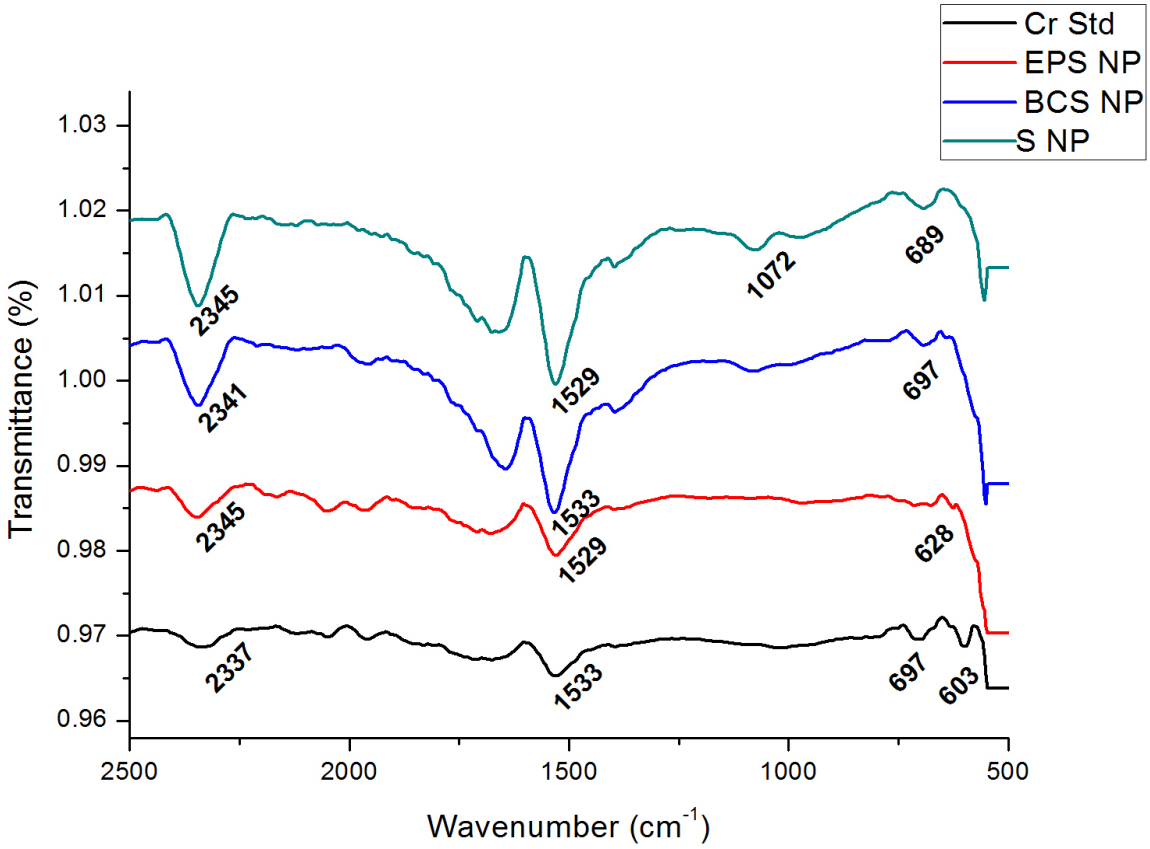


Fig 19. FTIR spectra of EPS NP, BCS NP (bacterial cell supernatant NP), SNP (supernatant NP) at 0.001 M Cr^{6+}

NPs were synthesized from bacterial cell pellet, supernatant and EPS with 0.01M Cr and FTIR data taken shows overlapping peaks at 1533 cm^{-1} in BCP NP & BS NP for 24h, 48h & 72h indicating presence of N-H stretch. NPs synthesized from bacterial cell pellet in 24h, 48h and

72h time interval shows overlapping peaks at 1533 cm^{-1} indicating presence of N-H stretch. (Fig 20) Peaks at 2341, 2353 cm^{-1} represent NH_3^+ in amino acids and peaks 685,632,681 cm^{-1} are observed in bacterial cell pellet NP after 24h, 48h and 72h respectively and peaks found at 632,619 cm^{-1} in BS NP 24 h and 72 h respectively indicating the formation of Cr_2O_3 NPs.

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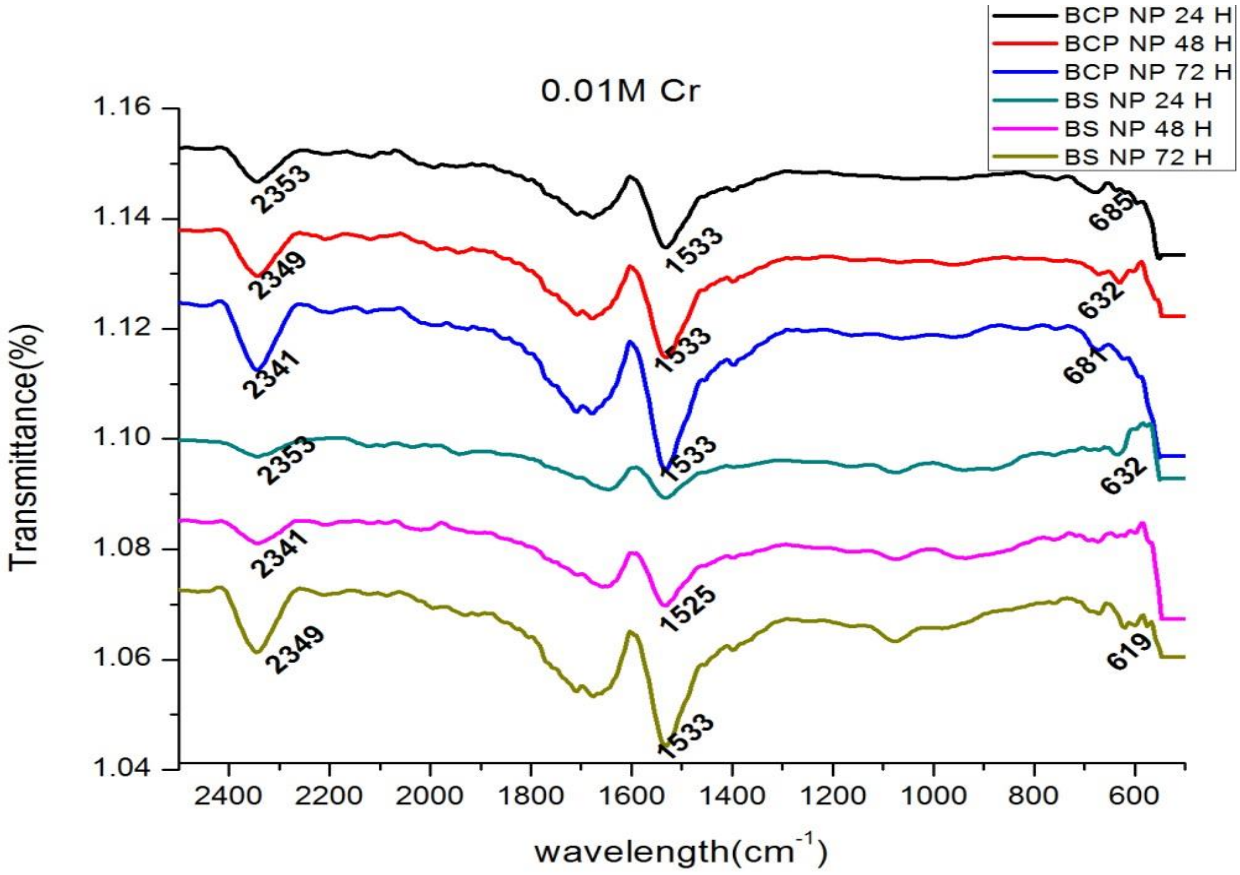


Fig 20. FTIR spectra of BS NP (bacterial supernatant NP) and BCP NP (bacterial cell pellet NP) obtained at 24, 48 and 72 h from 0.01 M Cr^{6+}

.

characteristic peaks were observed in 2345 cm^{-1} in supernatant NP pellet (SNP P) & supernatant nanoparticle supernatant (SNP S) representing NH_3^+ of amino acids(N-H stretching mode) due to as shown in Fig 21. and the peaks found at 619 in SNPS 628 in SNP P, 632 cm^{-1} in case of EPS NP and it may be due to presence of Cr_2O_3 interatomic vibration .

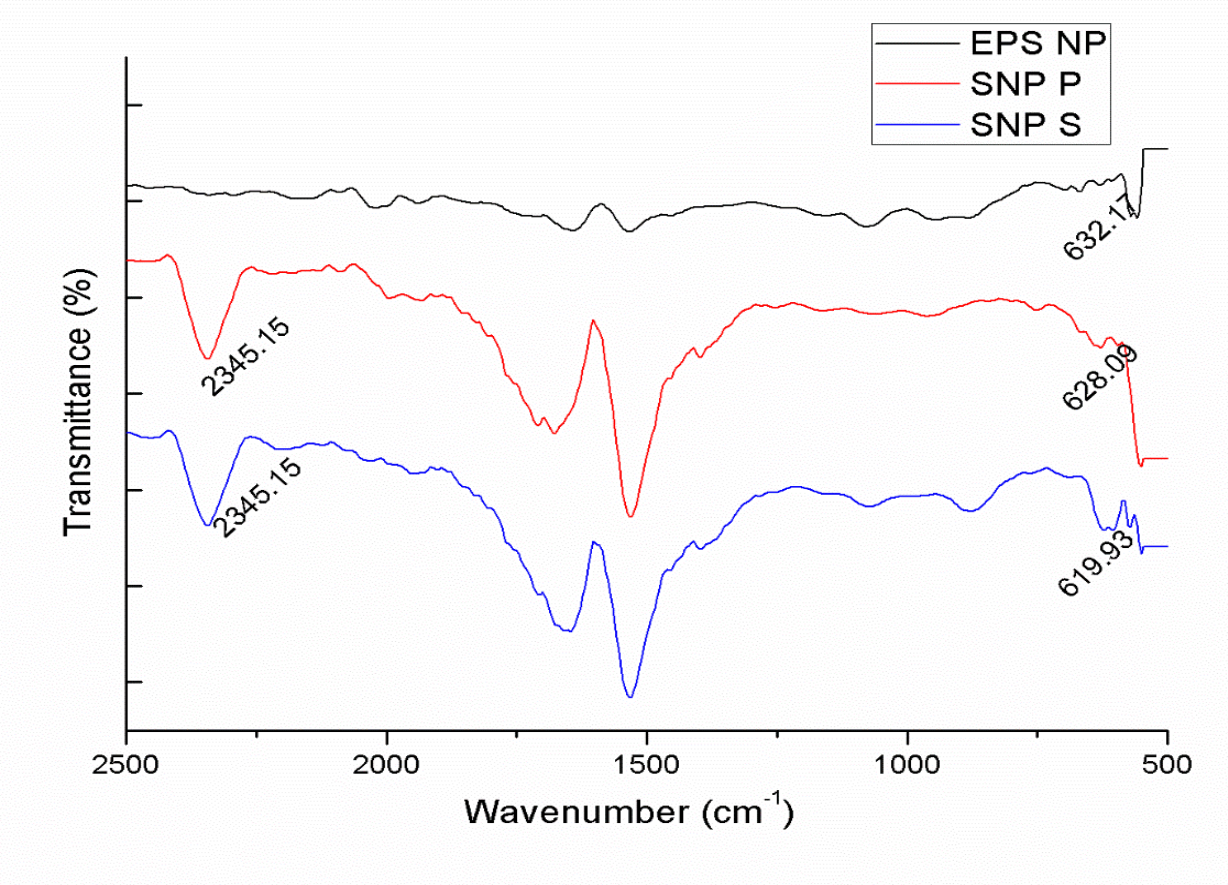


Fig 21. FTIR spectra of SNP P(Supernatant NP Pellet) ,SNP S(Supernatant NP Supernatant) &EPS NP obtained at 24, 48 and 72 h from 0.01 M Cr^{6+}

Zeta potential of Samples

Zeta potential indicates the nature of the electrostatic potential near the surface of a particle. Zeta potential of samples was taken to determine the charge on the particles, which determines the stability of the synthesized NPs.

Table 5. Zeta potential of control (without Cr supplemented)

Control	**Surface charge (mV)**
Bacterial Cell Pellet	25.2
Bacterial Supernatant	-7.95
EPS	-17.2

The zeta potential of control (bacterial cell pellet, bacterial supernatant and EPS) are presented in (Table 5) .Zeta potential of synthesized NPs were presented in graphical manner in Fig 22 for 0.01M Cr^{6+} and Fig 23 for 0.001M Cr^{6+}.

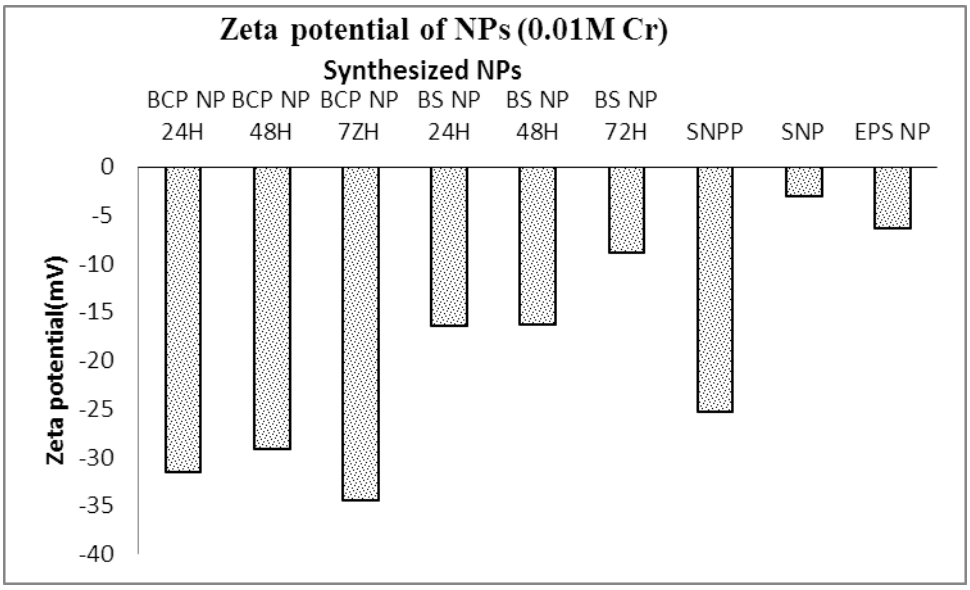


Fig 22. Zeta potential of different synthesized Nanoparticles (0.01M Cr^{6+})

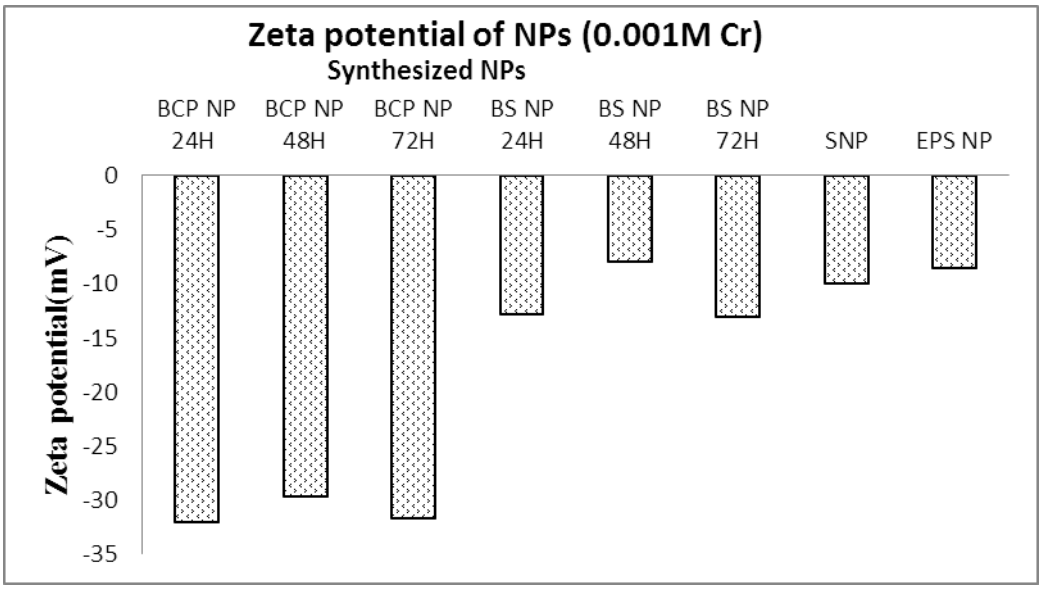
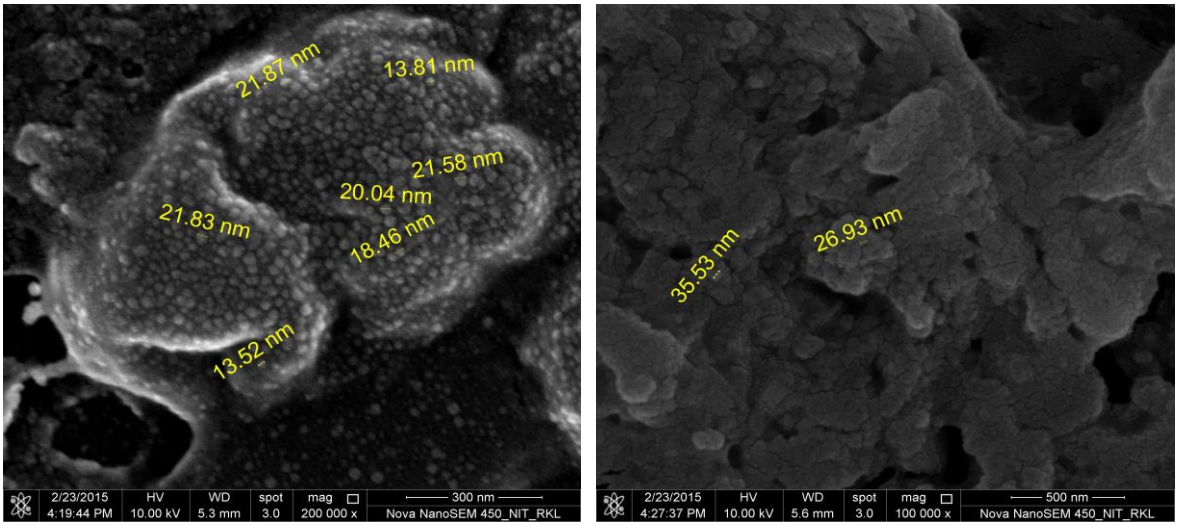


Fig 23. Zeta potential of different synthesized Nanoparticles (0.001M Cr^{6+})

Electron Microscopy

Scanning electron microscope of samples were taken in Nova Nano SEM field emission which confirms nanoparticle formation from bacterial cell pellet (BCP NP) and bacterial supernatant (BS NP).From the below Fig 24 we can see that there is synthesis of BCP NP of size 21.83 nm,18.46 nm, 20.04 nm ,13.81 nm etc. and BSNP of size 35.53 nm and 26.93 nm observed conforming the synthesis of Cr_2O_3 NPs.



a) b)

Fig 24. FESEM image of a) BCP NP (bacterial cell pellet NPs) b) BSNP (bacterial supernatant NPs) synthesized from 0.001M Cr^{6+}

EDX (Energy dispersive X-ray Spectroscopy):

EDX analysis of the synthesized NPs showed the presence of elemental Cr in it. (Fig 25)

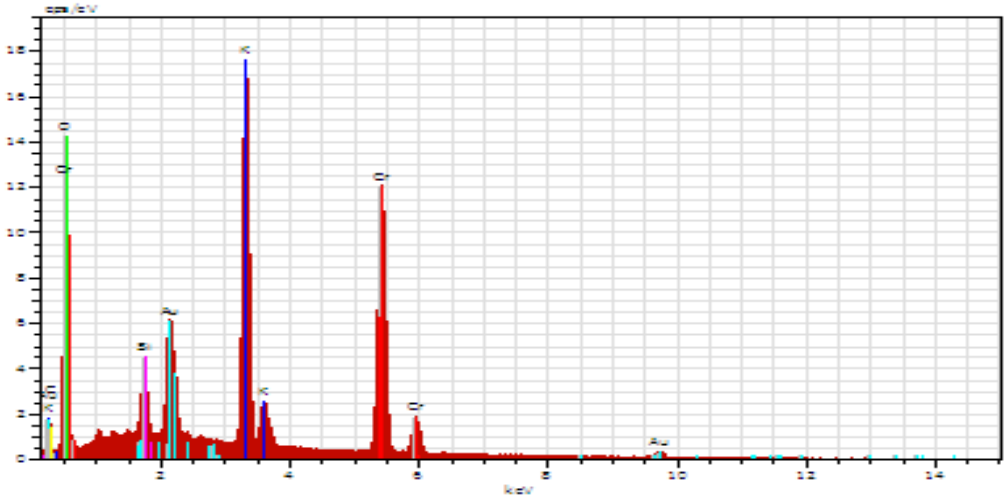


Fig 25. EDX analysis showing the presence of elemental Cr

Discussion

This 21st century world has led tremendous development leading to the comfort of people by exploiting the nature. Now the exploitation level has increased to such a high level that there is an urgent need to take care of it. Heavy metals are essential part of industrial pollution which has server adverse effect on human health. The conventional remediation strategies for acidic industrial effluents containing chromium involve physical and chemical methods which are costlier and site specific. However, previous reports of acidotolerant or acidophilic bacteria from chromium contaminated sites have given a hope to utilize these acidotolerant metal resistant bacteria for the remediation of contaminated sites (Navarro et al., 2013). Acidotolerant or acidophilic bacteria are classified under extremophiles which having adaptability to sustain and survive in diverse range of extreme environments including high metal stress (Banerjee, 2004).

Bioremediation or detoxification of Cr^{+6} by microorganisms have high applicability and low cost recovery of metal from contaminated sites. In this study we have attempted to reduce toxic hexavalent chromium (Cr^{+6}) into nontoxic trivalent chromium (Cr^{+3}) NPs by biofilm forming acidotolerant *Lysinibacillus* sp. RTA 01 bacterium. The isolated bacteria from chromium contaminated industrial effluent were screened on the basis of growth in low pH, high metal tolerance and biofilm forming ability of the strain. Selected bacterium *Lysinibacillus* sp. RTA 01 has significant growth in 5-6 pH and up to 250 ppm level of tolerance against Cr^{+6}. The growth condition studies of bacterium showed glucose and beef extract were favourable carbon and nitrogen sources among different sources The studied acidotolerant bacterium has also shown resistance to selected antibiotics; vancomycin, azithromycin, neomycin and tetracycline which suggested high adaptability and diverse defense mechanism (Davies et al., 2010). The biofilm screening of the bacteria in presence of metal confirmed influence of metal stress in the formation of biofilm as reported by Harrison et al., (2007). The extracellular polymeric substances (EPS) which have strong affinity towards metal ions and helps in metal adsorption (Hall-Stoodley et al. 2004). The FTIR analysis of EPS with varying concentration of Cr^{+6} have shown shift in metal interaction region of spectrum, which suggests EPS has metal binding ability as reported earlier (Bayramoglu et al. 2002). The results of chromium reduction assay through DPC method demonstrated that studied acidotolerant bacteria could reduce 53.94 % of Cr^{+6} into Cr^{+3} after 24 h of incubation at room temperature which has applicability in chromium detoxification (Focardi et al., 1992). To investigate the mechanism of chromium bioreduction by

Lysinibacillus sp. RTA 01, different part of the bacteria culture; cell mass (pellet), cell free media (supernatant) and extracellular polymeric substances (EPS) were incubated with two different concentration of Cr^{+6} (0.01M and 0.001M) and characterized further. The prominent colour change of the Cr^{6+} solution after incubation with bacterial part suggested reduction of bulk Cr^{+6} into Cr^{+3} NPs which was further confirmed by characteristics peaks in between 350 to 450 nm in UV-visible spectra (Annamalai et al., 2014; Meenambika et al., 2014). XRD analysis of the samples was also confirmed the presence of Cr metal in the samples with corresponding peaks at 24.2˚ and 28.25˚. FTIR analysis of the all synthesized NPs samples were taken to determine interaction of the functional groups with Cr metal ions. The presence of sharp peaks below 1000 cm^{-1} in the range of 600-710 cm^{-1} suggest possible metal interaction and interatomic vibration of Cr_2O_3 (Quintelas and Tavares, 2001). To further confer the size of the NPs which were synthesized by bacterial cell mass (pellet) and cell free media (supernatant) electron microscopy (FESEM) was performed. The presence of 13 nm to 35 nm sized spherical Cr^{+3} NPs were observed and further confirmed by electron dispersive spectra (EDX) of samples. The surface charge analysis of all the synthesized NPs by have high negative values which suggested their high stability in aqueous media compared to previous reports of biosynthesis of Cr_2O_3 NPs. (Annamalai et al., 2014).

The present work illustrated the complete biological synthesis of Cr^{+3} NPs by the bacterial strain which further increases the adsorption capacity of the isolate and help in remediation of chromium contaminated sites sustainably.

Conclusion

Gram positive rod shaped bacterial isolate *Lysinibacillus* sp. RTA 01 producing biofilm-EPS showed high level of metal resistance (250 ppm) against hexavalent chromium along with other heavy metals (Pb^{2+} Zn^{2+} Cd^{2+}). Acidotolerant nature of the isolate helps it to survive and have significant growth in acidic environment. The isolate showed high chromate reduction capacity that can efficiently reduce 53.94% Cr^{6+} from the aqueous solution. The UV- Visible spectra of reduced chromate showed characteristic peaks of Cr^{+3} NPs, which were further confirmed in XRD analysis, FESEM and EDX. FTIR spectra also confirmed significant interaction of cell mass and EPS with metal ion, which was also presented in Zeta potential analysis of synthesized NPs. This bioreduction of Cr^{6+} is a reliable, ecofriendly and nontoxic technique which reduced the clean-up time, can be widely utilized in management of industrial acidic toxic wastes containing Cr^{6+}. The acidotolerant bacterium *Lysinibacillus* sp. RTA 01 not only has better chromate reduction capacity but also has shown additional benefits like synthesis of economically important Cr^{+3} NPs with easy recovery, easy synthesis, and absence of secondary pollutant, cost-effectiveness and environmental-friendly process. Thus, by further advancement of biotechnological and nanotechnological approaches, removal of different heavy metals from environment could be improved by enhanced adsorption and recovery.

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